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Immediate up-regulation of the calcium-binding protein S100P and its involvement in the cytokinin-induced differentiation of human myeloid leukemia cells

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Abstract

Cytokinins are important purine derivatives that act as redifferentiation-inducing hormones to control many processes in plants. Cytokinins such as isopentenyladenine (IPA) and kinetin are very effective at inducing the granulocytic differentiation of human myeloid leukemia HL-60 cells. We examined the gene expression profiles associated with exposure to IPA using cDNA microarrays and compared the results with those obtained with other inducers of differentiation, such as all-*trans* retinoic acid (ATRA), 1α,25-dihydroxyvitamin D_3 (VD3) and cotylenin A (CN-A). Many genes were up-regulated, and only a small fraction were down-regulated, upon exposure to the inducers. IPA and CN-A, but not ATRA or VD3, immediately induced the expression of mRNA for the calcium-binding protein S100P. The up-regulation of S100P was confirmed at the protein expression level. We also examined the expression of other S100 proteins, including S100A8, S100A9 and S100A12, and found that IPA preferentially up-regulated S100P at the early stages of differentiation. IPA-induced differentiation of HL-60 cells was suppressed by treatment with antisense oligonucleotides against S100P, suggesting that S100P plays an important role in cell differentiation.

Keywords: Myeloid leukemia; Differentiation; S100P; Calcium-binding protein

1. Introduction

Cytokinins are important purine derivatives that served as hormones that control many processes in plants. Cytokinins induce callus to redifferentiate into adventitious buds. Callus are clusters of dedifferentiated plant cells that are immortal and proliferate indefinitely in a disorganized manner, just like human cancer cells. Because there are some similarities in the biological phenotypes of cancer cells and callus cells, cytokinins may also affect the differentiation of human cancer cells. Cytokinins are found in uterine taken from both patients with lung carcinoma and healthy subjects [1]. These results suggest that cytokinins and their derivatives may play some roles on human normal and malignant cells. We found that cytokinins and their derivatives effectively inhibit the proliferation and induce the granulocytic differentiation of human myeloid leukemia cells, although the differentiation can be induced by retinoids, vitamin D_3, cytokines and various chemicals [1–3].

There may be common final pathways that mediate maturation in malignant cells, but the precise mode of action of differentiation inducers may differ between these inducers. We previously studied the mechanism of action of cytokinins in human myeloid leukemia HL-60 cells [4]. The metabolism of cytokinins to their nucleotides was

Abbreviations: IPA, isopentenyladenine; ATRA, all-*trans* retinoic acid; VD3, 1α,25-dihydroxyvitamin D_3; CN-A, cotylenin A; MAPK, mitogen-activated protein kinase; NBT, nitroblue tetrazolium

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closely associated with cytokinin-induced differentiation and growth inhibition, but cytokinins were not incorporated into RNA and DNA [4]. These results suggest that cytokinin nucleotides themselves play an important role in inducing the differentiation of HL-60 cells. Cytokinins effectively induced a phosphorylated (active) form of mitogen-activated protein kinase (MAPK). MAPK activation was necessary for cytokinin-induced differentiation [4]. A more comprehensive understanding of the molecular mechanism of cytokinin action on leukemia cells may provide information to improve differentiation therapy against myeloid leukemia. The present study was undertaken to evaluate the gene expression profiles associated with cytokinin-induced differentiation in comparison with those using other differentiation inducers.

2. Materials and methods

2.1. Materials

Isopentenyladenine (IPA), RPMI 1640 medium, nitroblue tetrazolium (NBT), all-*trans* retinoic acid (ATRA) and anticancer drugs were purchased from Sigma Chemical Co. (St. Louis, MO), while 1α,25-dihydroxyvitamin D_3 (VD3), methyl jasmonate and dimethyl sulfoxide were purchased from Wako Chemicals (Osaka, Japan). Cotylenin A (CN-A) was prepared as described previously [5]. PD98059 was obtained from Calbiochem (La Jolla, CA).

2.2. Cells and cell culture

The HL-60 cell line, derived from an AML patient, U937 and THP-1 monoblastic leukemia cells and NB4 promyelocytic leukemia cells were maintained in an RPMI 1640 medium supplemented with 10% fetal bovine serum and 80 μg/ml gentamicin at 37 °C in a humidified atmosphere of 5% CO_2 in air. In experiments where cells were treated with PD98059, cells were cultured with the inhibitor 16 h before the start of measurements. At 0 h, PD98059-treated cells were cultured with differentiation inducers, and the same concentration of PD98059 was again added to the cultures. At 16 h, PD98059 was added again, and these three successive additions of PD98059 had no apparent growth-inhibitory effect.

2.3. Assay of cell growth and properties of differentiated cells

Suspensions of cells (5×10^4 cells/ml) in 2 ml of culture medium were incubated with or without the test compounds in multidishes (Costar, Cambridge, MA). Cell numbers were counted in a Model ZM Coulter Counter (Coulter Electronics, Luton, UK). Superoxide-generating oxidase was determined by the ability of the cells to reduce NBT upon exposure to 12-*O*-tetradecanoyl phorbol-13-acetate. Cells were incubated in 1 ml of RPMI 1640 medium containing NBT (1 mg/ml) and 12-*O*-tetradecanoyl phorbol-13-acetate (100 ng/ml) at 37 °C for 50 min. The reaction was stopped by adding 5 M HCl (1 M, final concentration). The suspension was kept at room temperature for 20 min and then centrifuged. Formazan deposits were solubilized in dimethyl sulfoxide, and the absorption of the formazan solution at 560 nm/10^7 cells was measured in a spectrophotometer. Morphological changes were examined in cell smears stained with May-Grünwald-Giemsa solution.

2.4. cDNA microarray analysis

Total RNA was isolated from HL-60 cells treated with or without inducers for 4, 12, and 24 h using Isogen (Nippon Gene, Toyama, Japan). Poly(A)$^+$RNA was reverse-transcribed with the concomitant incorporation of Cy3- and Cy5-labeled nucleotides. The labeled probes were hybridized with a cDNA microarray representing about 1000 different human genes specified for human cancer (TaKaRa Bio Inc., Tokyo), and their fluorescent intensities were scanned according to the protocol standardized by TaKaRa Bio Inc. Twelve housekeeping genes were spotted onto each slides and used for signal normalization. The genes were screened by analyzing the difference in expression profiles between two genes. All the analyses were done according to the instructions of TaKaRa Bio Inc.

2.5. Gene expression analysis by RT-PCR

Total RNA was extracted using Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Total RNA (1 μg) from leukemia cells was converted to first-strand cDNA primed with random hexamer in a reaction volume of 20 μl using an RNA PCR kit (TaKaRa Bio), and 4 μl of this reaction was used as a template in the polymerase chain reaction. The oligonucleotides used in PCR amplification were as follows: sense strand, 5′-AATCTAGCACCATGACGGAA-3′, and antisense strand, 5′-CAACAAACACTTTTGGGAAG-3′, for S100P; sense strand, 5′-AGAAAGCCTTGAACTCTATC-3′, and antisense strand, 5′-TACTCTTTGTGGCTTTCTTCAT-3′, for S100A8; sense strand, 5′-ATGACTTGCAAAATGTCGCA3′, and antisense strand, 5′-ATCTTGGCCACTGTGGTCTTA-3′, for S100A9; sense strand, 5′-ACTGCTGGCTTTTTGCTGTA-3′, and antisense strand, 5′-AGTGTGTTTATTAACTCTTA-3′, for S100A12; sense strand, 5′-ATGGCTCTCAGAGTCCTTCT-3′, and antisense strand, 5′-GTGTTCTCACTGCAAGTCTG, for CD11b; sense strand, 5′-GGTCGGAGTCAACGGATTTG-3′, and antisense strand, 5′-ATGAGCCCCAGCCTTCTCCAT-3′, for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR consisted of 25 cycles for S100P, 30 cycles for S100A8, 23 cycles for S100A9, 28 cycles for S100A12, 32 cycles for CD11b and 18 cycles for GAPDH, with

denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1.5 min. The quantitative RT-PCR reaction was performed as described in the literature [6].

2.6. Western blotting

The cells were packed after washing with cold phosphate-buffered saline, and then suspended at a concentration of 5×10^8 cells/ml in lysis buffer [20 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 μg/ml leupeptin, 1 mM PMSF]. The lysates were centrifuged at 105,000×*g* for 60 min and the supernatants were then resolved on 10–20% gradient SDS-polyacrylamide gels. The proteins were transferred electrophoretically from gel to an Immobilon-P membrane (Millipore, Bedford, MA, USA) and immunoblotted with anti-S100P (BD Biosciences, San Jose, CA) and anti-actin (Sigma) antibodies. Alkaline phosphatase-conjugated IgG (Bio-Rad Laboratories, Hercules, CA, USA) was used as a secondary antibody (1:2000 dilution). The bands were developed by treatment with the Immune-Star™ AP chemiluminescent (Bio-Rad Laboratories, Hercules, CA, USA) for 3 min at room temperature, and detected using a Fuji Lumino Image Analyzer LAS-1000 system (Fuji Film, Co. Ltd., Tokyo).

2.7. Antisense oligonucleotides and cell treatment

Phosphorothioate and morphilino oligonucleotides (18 mers) corresponding to the antisense and missense sequences flanking the translation initiation region of human mRNA for S100P were prepared by TaKaRa Bio Inc. and Gene Tools, LLC (Philomath, OR), respectively. The sequences were as follows; S100P, TGTCTCTAGTTCCGTCAT, and missense-S100P, TCTCTCAACTTCGGTCTT. Treatment with Morphilino oligonucleotides was performed according to the manufacturer's instructions. Phosphorothioate oligonucleotides were treated using Tfx™ reagent (Promega, Madison, WI). The Tfx™ reagent was suspended with distilled water the day before transfection and stored at −20 °C. On the day of transfection, the Tfx™ reagent/DNA mixture was prepared. In a sterile tube, 0–20 μl of DNA from a 500 μM stock solution and 0–40 μl of Tfx™ reagent were added to a total volume of 0.85 ml serum-free medium (prewarmed to 37 °C). The ratio of Tfx™ reagent to DNA was 2 to 1. The mixtures were incubated 10–15 min at room temperature. A 0.5 ml aliquot of cells (4×10^5 cells in serum-free medium) was put into each well of a 24-well plate. The mixtures were mixed and added to the wells (0.2 ml/well). After 12 h, 0.2 ml of serum-free medium, 0.1 ml of serum, a differentiation inducer and the oligomers were added to each well.

2.8. Statistical evaluation

Statistical analyses were performed using an unpaired two-tailed Student's *t*-test.

3. Results

3.1. cDNA microarray analysis of HL-60 cells treated with IPA, CN-A, ATRA and VD3

IPA is a potent cytokinin and also a potent inducer of the differentiation of human myeloid leukemia cells [1]. CN-A, which has cytokinin-like activities and was isolated as a plant growth regulator, is also a potent inducer [3,7]. When HL-60 cells were cultured with 25 μg/ml of IPA, 10 μg/ml of CN-A, 400 nM ATRA or 24 nM VD3 for 3 days, NBT reduction was induced at the same levels (1.12–1.35 $A_{560}/10^7$ cells), suggesting that these treatments similarly induce the differentiation of HL-60 cells. Three individual preparations were independently subjected to hybridization. A good correlation was observed among the duplicate spots, and the net ratios for the housekeeping genes were between 0.89 and 1.1. Genes with a treatment:control ratio >2 in all three experiments were considered to be significantly up-regulated and those with a treatment:control ratio <0.5 were considered to be significantly down-regulated. The changes in the gene expression of HL-60 cells treated by IPA, CN-A, ATRA or VD3 for 24 h are shown in Table 1. IPA, CN-A, ATRA and VD3 significantly up-regulated the expression of 15, 21, 16, and 4 genes, respectively. All the inducers significantly induced the expression of tumor necrosis factor-α-induced protein 1 mRNA. VD3 greatly induced the expression of the arachidonate 5-lipoxygenase and prostaglandin-endoperoxide synthase 1 genes, whereas IPA did not significantly affect the expression of these genes (Table 1). The results indicate that IPA induces a different gene expression profile than that induced by ATRA, although both inducers stimulate the granulocytic differentiation of HL-60 cells. The gene profile in cells treated with CN-A has features of those using IPA and ATRA. The gene for the calcium-binding protein S100P was the most up-regulated gene in cells treated with IPA and CN-A. RAB31 and arachidonate 5-lipoxygenase were the most up-regulated in the ATRA- and VD3-treated cells, respectively. The time course of the expression of S100P mRNA revealed that IPA and CN-A similarly and rapidly induced the expression of this gene in HL-60 cells (Fig. 1A), suggesting that the rapid induction of the expression of this gene is involved in the induction of differentiation induced by IPA and CN-A. IPA up-regulated the expression of the regulator of G-protein signaling-16 (RGS16) gene, while ATRA significantly down-regulated the expression of this gene. The changes were also observed in cells treated for 4 h (Fig. 1B). When the cells were treated with these inducers for 24 h, only 4 or 5 genes were down-regulated (Table 1). The expression of the gene for non-receptor type 3 protein tyrosine phosphatase was greatly reduced by the inducers, although the amounts of RNA in the treated cells were below reliable levels. Transcription factor Dp-2 was constantly down-regulated by these inducers, even with treatment for 12 h (data not shown).

Table 1
Changes in levels of genes in HL-60 cells after exposure to IPA, CN-A, ATRA or VD3 for 24 h

Gene name	Gene expression ratio			
	IPA	CN-A	ATRA	VD3
Up-regulated				
S100 calcium-binding protein P	**6.86**	**7.77**	1.69	1.24
phorbol-12-myristate-13-acetate-induced protein 1	**4.55**	1.71	1.91	1.41
phosphodiesterase 4B, cAMP-specific	**4.43**	**3.89**	1.3	0.81
Clk-associating RS-cyclophilin	**3.56**	**3.98**	1.51	1.44
tumor necrosis factor, alpha-induced protein 2	**3.41**	**2.89**	**3.59**	**2.46**
jagged1 (Alagille syndrome)	**3.32**	1.71	0.85	1.64
B-cell translocation gene 1, anti-proliferative	**3.21**	**2.48**	**2.61**	1.44
T cell receptor alpha locus	**3.11**	1.85	0.53	n.e.
thioredoxin reductase 1	**2.91**	1.5	0.93	1.31
Ric (*Drosophila*)-like, expressed in many tissues	**2.83**	1.74	1.31	0.96
Pirin	**2.76**	1.51	0.6	0.83
regulator of G-protein signalling 16	**2.73**	1.3	0.37	0.79
KIAA0410 gene product	**2.42**	1.41	0.87	0.95
asparagine synthetase	**2.31**	1.53	0.61	1.12
protein tyrosine phosphatase, non-receptor type 12	**2.04**	1.97	1.38	0.98
dual specificity phosphatase 6	1.84	**2.28**	**3.11**	1.09
annexin A1	1.74	**2.06**	1.72	1.12
arachidonate 5-lipoxygenase	1.67	**3.94**	**3.06**	**7.13**
protein tyrosine phosphatase, receptor type, c polypeptide	1.45	**3.02**	**2.32**	1.12
CD48 antigen (B-cell membrane protein)	1.44	**2.59**	1.68	0.72
bone marrow stromal cell antigen 1	1.44	**2.05**	1.53	1.96
spermidine/spermine N1-acetyltransferase	1.41	1.55	**2.15**	1.77
natural killer cell transcript 4	1.33	**2.17**	1.01	0.86
RAB31, member RAS oncogene family	1.33	**2.14**	**6.21**	1
E74-like factor 4 (ets domain transcription factor)	1.32	1.83	**2.17**	1.33
LIM and SH3 protein 1	1.26	**2.07**	**2.03**	0.99
pleckstrin	1.24	1.71	**2.98**	1.49
FYN-binding protein (FYB-120/130)	1.19	1.88	**2.03**	0.85
putative gene product	1.14	**2.97**	0.97	0.87
protein kinase C binding protein 2	1.13	**2.17**	0.62	0.88
HSPC022 protein	1.13	**2.11**	1.53	1.15
KIAA1024 protein	1.12	1.79	1.04	**2.22**
inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	1.1	1.33	**2.11**	n.e.
tissue inhibitor of metalloproteinase 2	1.05	**3.88**	**2.57**	1.06
lectin, galactoside-binding, soluble, 3 binding protein	1.02	**2.09**	**4.52**	0.86
interferon, alpha-inducible protein (clone IFI-6-16)	1.01	**2.3**	**2.96**	1
prostaglandin-endoperoxide synthase 1	0.97	**2.85**	**3.73**	**5.19**
Down-regulated				
protein tyrosine phosphatase, non-receptor type 3	0.22*	0.18*	0.4	0.19*
vimentin	0.36	0.98	0.23	0.36
transcription factor Dp-2 (E2F dimerization partner 2)	0.43	0.31	0.4	0.39
guanine nucleotide binding protein (G protein), beta 5	0.44	0.78	0.8	0.5
aldehyde dehydrogenase 5	0.71	0.48	0.95	0.77
v-ets avian erythroblastosis virus E26 oncogene homolog 2	0.85	0.43	0.62	0.52
Rab9 effector p40	1.07	0.44	0.75	0.86
interleukin 2 receptor, gamma (severe combined immunodeficiency)	1.85	1.01	0.71	0.37
argininosuccinate synthetase	1.88	1.19	0.41	1.15
regulator of G-protein signalling 16	2.73	1.3	0.37	0.79

Values are means of three separate experiments and values in half-toned boxes indicate significantly changed genes.

3.2. Regulation of S100P gene expression

To confirm the results from the cDNA microarray analysis, the expression of S100P was examined by quantitative RT-PCR analysis. When HL-60 cells were treated with IPA or CN-A for 24 h, the accumulation of S100P transcript was greatly induced. On the other hand, treatment with ATRA or VD3 did not significantly affect the expression of the mRNA (Fig. 2A and B). S100P is a member of S100 calcium-binding protein family, which is mainly present in human placenta [8,9]. The S100 family includes 19 members that are differentially expressed in a large number of cell types and have been implicated in the regulation of various intracellular activities [10,11]. Pre-

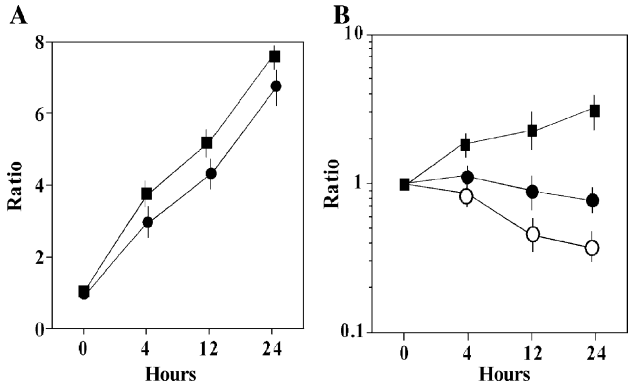

Fig. 1. Time-course of S100P and RGS16 mRNA expression in HL-60 cells treated with differentiation inducers. (A) Effects of CN-A and IPA on S100P mRNA expression. Cells were treated with 25 μg/ml IPA (■) or 10 μg/ml CN-A (●) for 4, 12 and 24 h. (B) Effects of IPA and ATRA on RGS16 mRNA. Cells were treated with 25 μg/ml IPA (■), 10 μg/ml CN-A (●) or 400 nM ATRA (○). Data represent the mean±S.D. of three determinations.

vious studies have shown that some members of the S100 family such as S100A8, A9 and A12 are up-regulated in neutrophils and monocytes during inflammatory responses [12–14]. S100A8 and A9 exhibit potent chemoattractive activity for leukocytes, and have been suggested to affect the cytoskeleton and cell shape [15–17]. These results suggest that the gene expression of these family members might be affected during the differentiation of HL-60 cells. Fig. 2C shows that treatment with IPA or CN-A for 24 h preferentially up-regulates the gene expression of S100P. This up-regulation by IPA was observed within 3 h (Fig. 3A), which confirms the results of the cDNA microarray analysis (Fig. 1). We also examined whether the induction by IPA was limited to the RNA level or extended to differential protein expression. IPA and CN-A led to increases in the S100P protein level as assayed by Western blotting, whereas ATRA or VD3 did not significantly increase the protein level (Fig. 3B). The results confirmed those obtained by RT-PCR (Fig. 2A). Although treatment with IPA for 24 h hardly affected the expression of the mRNA for S100A8 and S100A9, up-regulation was observed in the cells treated for 3 days (Fig. 4). Treatment with ATRA or VD3 for 3 days up-regulated the expression of the S100A8 and A9 genes. The induction of S100P mRNA was also observed in cells that had been treated with ATRA for 3 and 5 days, whereas VD3 did not stimulate the expression of S100P mRNA (Fig. 4). These results indicate that IPA and CN-A immediately and preferentially stimulate expression of the S100P gene.

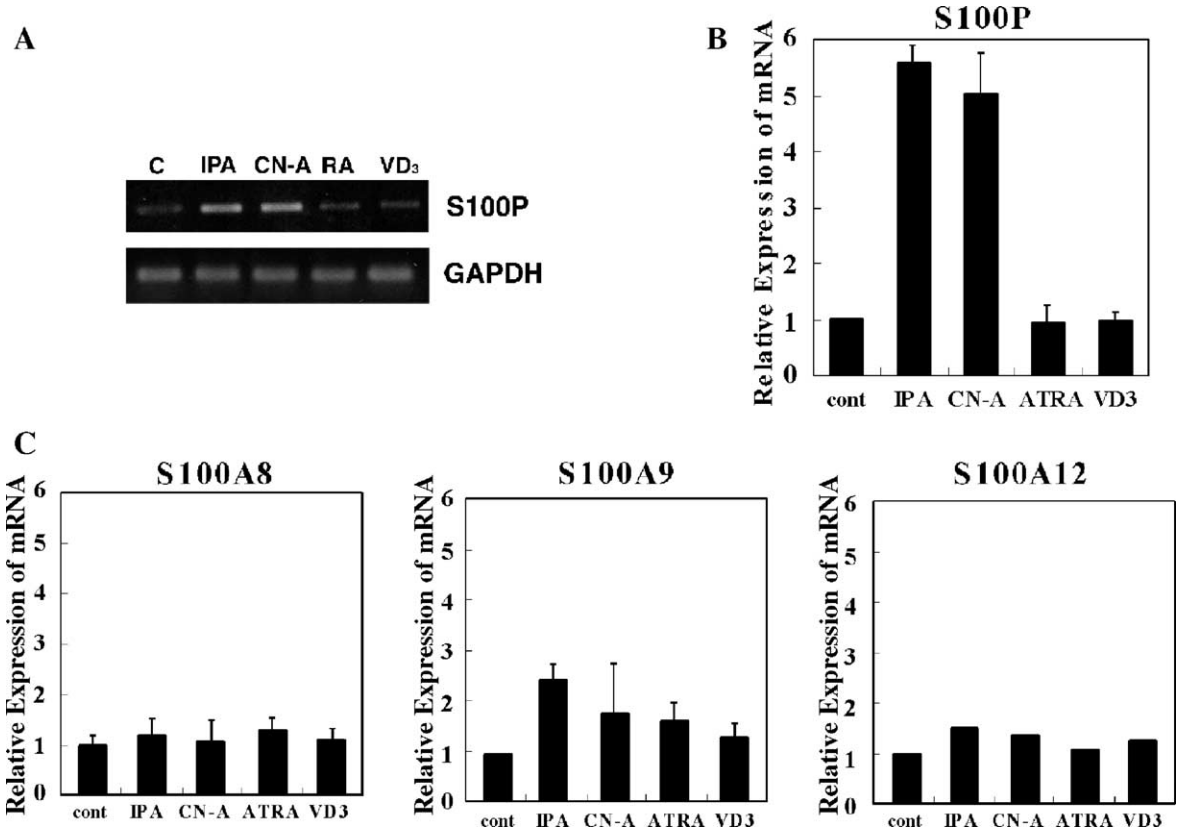

Fig. 2. Effects of differentiation inducers on the expression of S100 family members. (A) The expression of S100P mRNA was examined by RT-PCR analysis. HL-60 cells were untreated or treated with 25 μg/ml IPA, 10 μg/ml CN-A, 400 nM ATRA, or 24 nM VD3 for 4 h. The levels of GAPDH expression are shown to demonstrate that equal amounts of RNA were used for RT-PCR. The mRNA expression of S100P (B) and other members of the S100 family (C) was determined by RT-PCR. HL-60 cells were untreated or treated with 25 μg/ml IPA, 10 μg/ml CN-A, 400 nM ATRA, or 24 nM VD3 for 1 day. Graph depicts relative levels of mRNA after normalizing to GAPDH mRNA levels. Data represent the mean±S.D. of three determinations.

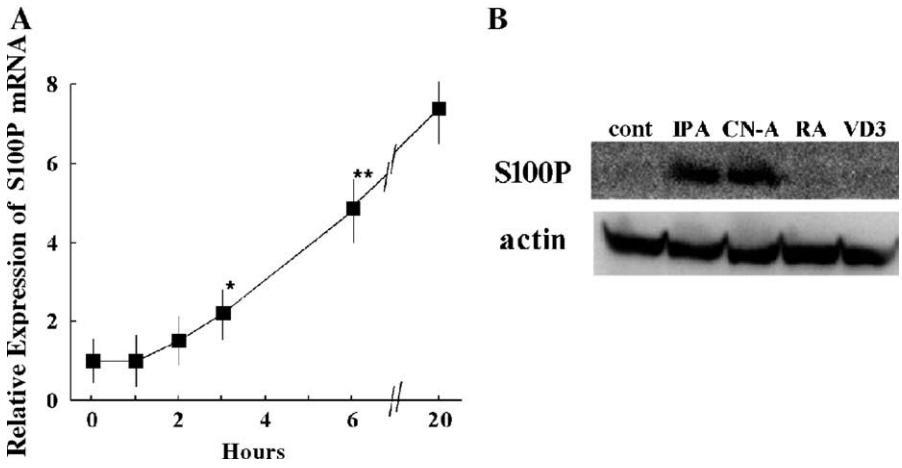

Fig. 3. Induction of S100P mRNA and protein by IPA. (A) Time-course of S100P mRNA expression induced by 25 μg/ml IPA. Values are means±S.D. for three separate experiments. *P<0.05, **P<0.01. (B) Induction of S100P protein in cells treated with IPA or CN-A for 2 days. Treatments with the inducers were as in Fig. 2A.

To confirm the preferential up-regulation of S100P mRNA during early differentiation induced by IPA or CN-A, the effects of various compounds on S100P mRNA expression were examined and compared to their growth-inhibitory activities (Fig. 5A). Methyl jasmonate (MJ), a plant stress hormone, can induce the differentiation of HL-60 cells [18]. This inducer also up-regulated gene expression, while other inducers, such as dimethyl sulfoxide and actinomycin D, did not (Fig. 5A). Aphidicolin and hydroxyurea effectively inhibited cell growth, but these drugs did not affect expression of the S100P gene. These results suggest that some, but not all, inducers stimulate the immediate up-regulation of S100P gene expression.

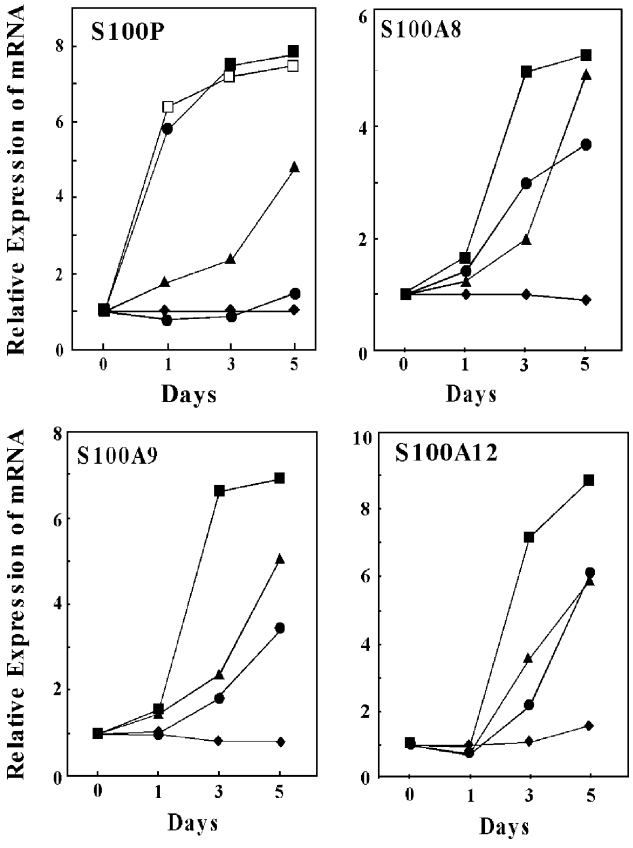

Fig. 4. Regulation of mRNA expression of members of the S100 family in HL-60 cells by various inducers. Cells were treated without (♦) or with 25 μg/ml IPA (■), 10 μg/ml CN-A (□), 400 nM ATRA (▲), or 24 nM VD3 (●) for 1, 3 and 5 days. Values are means of three separate experiments.

The up-regulation of S100P gene expression by IPA was also observed in other myeloid leukemia cells such as NB4, THP-1 and U937 cells, suggesting that up-regulation is not limited to HL-60 cells (Fig. 5B).

3.3. Relationship between S100P gene expression and the induction of differentiation

We previously demonstrated that IPA significantly increased mitogen-activated protein kinase (MAPK) activity within 2 h, this enhancement persisted for 24 h in HL-60 cells, and the enhancement induced by IPA was more prominent than that induced by ATRA [4]. The increased MAPK activity was reduced by pretreatment with PD98059, an inhibitor of MAPK kinase (MEK). This inhibitor also suppressed the NBT reduction induced by IPA (Fig. 6A). Treatment with PD98059 reduced the expression of S100P mRNA and inhibited differentiation (Fig. 6B), suggesting that the expression of this gene is associated with IPA-induced differentiation.

To understand whether the expression of S100P is directly correlated with IPA-induced differentiation, cells were treated with antisense oligonucleotides for S100P to inhibit expression. HL-60 cells were treated with IPA in the presence or absence of the antisense oligomer, and the effects on growth and NBT reduction were observed (Fig. 7A and B). The growth-inhibitory effect caused by more than 20 μg/ml of IPA was significantly prevented by pre-incubation with antisense oligomer for S100P (Fig. 7A).

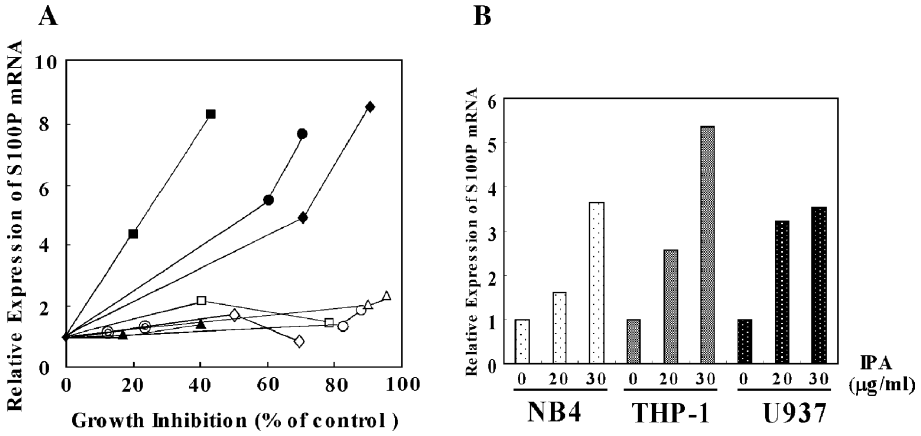

Fig. 5. Growth inhibition and the expression of S100P mRNA in HL-60 cells treated with various compounds (A), and induction by IPA of S100P mRNA in other leukemia cells (B). (A) HL-60 cells were cultured with various concentrations of IPA (●), MJ (■), cotylenin A (♦), ATRA (▲), VD3 (◎), dimethyl sulfoxide (◇), actinomycin D (□), hydroxyurea (○) and aphidicolin (△) for 1 day. The graph depicts relative levels of S100P mRNA after normalizing to GAPDH mRNA levels. Values are means of three separate experiments. (B) Cells were treated with various concentrations of IPA for 1 day. Values are means of three separate experiments.

Such pre-treatment also prevented the induction of NBT reduction by IPA (Fig. 7B). HL-60 cells treated with the antisense oligomer significantly reduced the IPA-induced expression of S100P mRNA compared with the control cells (Fig. 7C). The expression of mRNA for CD11b, a typical marker of the differentiation of HL-60 cells, was also prevented by the antisense oligomer (Fig. 7D), whereas the missense oligomer had no effect, indicating that the effects of antisense oligomer are specific. Similar results were obtained when we examined the morphological changes in HL-60 cells treated with IPA in the presence or absence of the antisense oligomer (Fig. 7E and F). Morphological differentiation of HL-60 cells treated with 25 μg/ml IPA into granulocytes was completely prevented by treatment with the antisense oligomer. The antisense oligomer to S100P hardly affected the ATRA- or VD3-induced differentiation of HL-60 cells (data not shown). These results suggest that S100P plays a crucial role in the IPA-induced differentiation of myeloid leukemia cells.

4. Discussion

Both IPA and ATRA can induce HL-60 cells to differentiate into mature neutrophils, but the gene expression profiles are quite different. The expression of the RGS16 gene was significantly enhanced by IPA, whereas ATRA significantly suppressed this gene expression. On the other hand, RAB31 mRNA was the most up-regulated among the genes in cells treated with ATRA, but was not significantly affected by IPA (Table 1). The up-regulation of RAB31 in ATRA-treated HL-60 cells was also demonstrated by Lee et

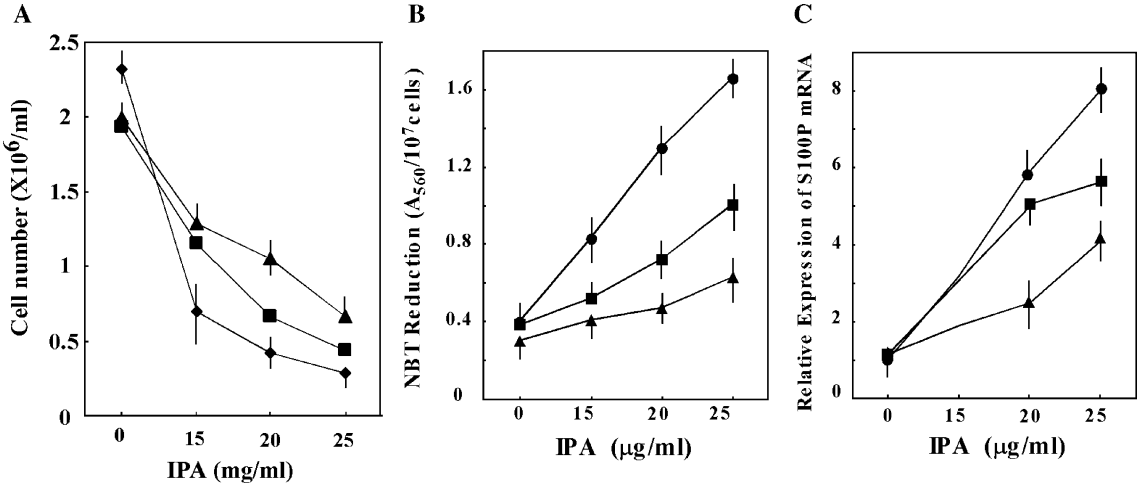

Fig. 6. Effects of PD98059 on IPA-induced growth inhibition (A), NBT reduction (B) and S100P mRNA expression (C) in HL-60 cells. Cells were cultured with various concentrations of IPA in the absence (●) or presence of 1.3×10^{-7} M (■) and 2.6×10^{-7} M (▲) PD98059 for 4 days. (C) Total RNA was isolated from an aliquot of cells used in Panel (A), and expression levels were determined by RT-PCR analysis. Graph depicts relative levels of S100P mRNA after normalizing to GAPDH mRNA levels.

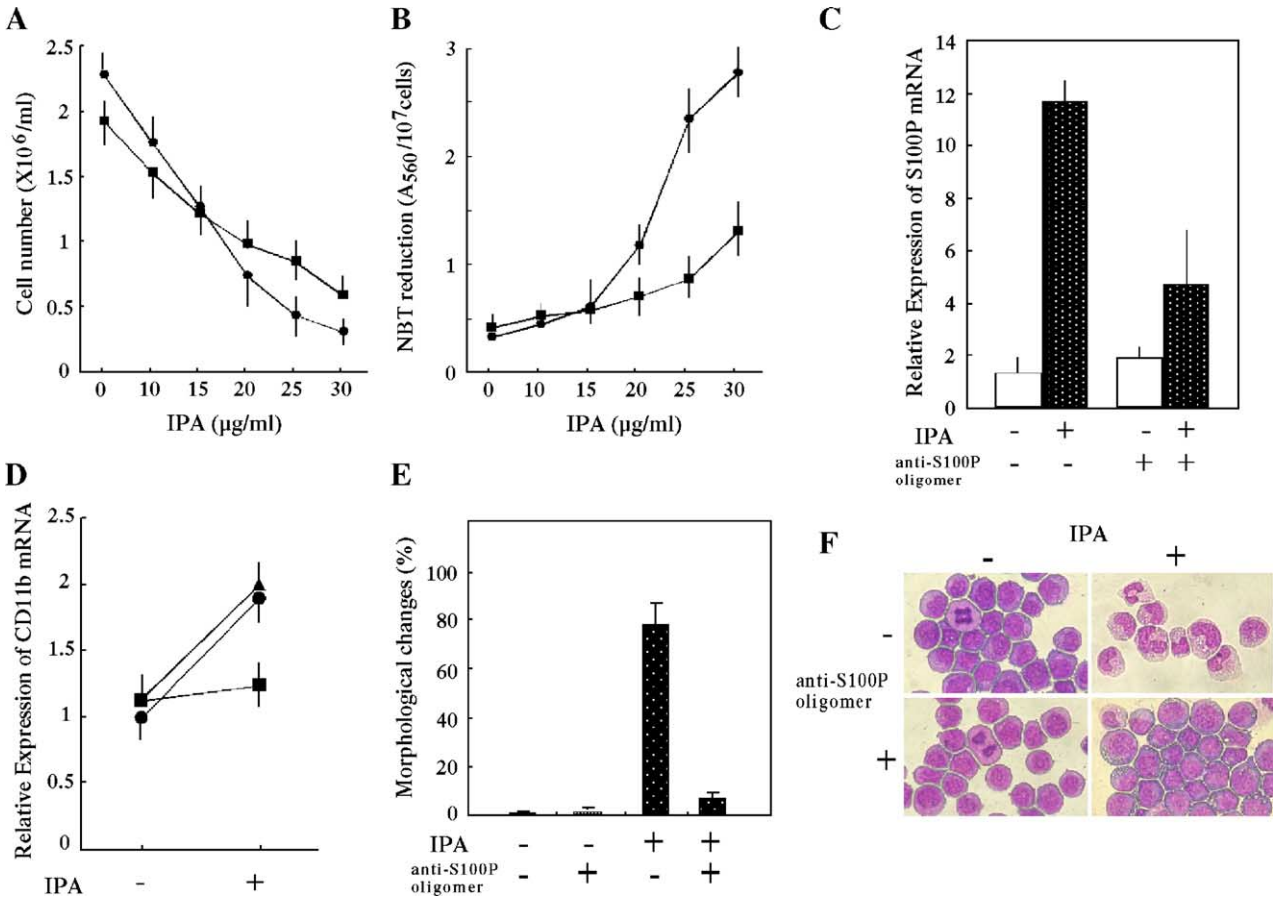

Fig. 7. Effects of antisense oligonucleotides for S100P on the growth inhibition and differentiation of HL-60 cells induced by IPA. Cells were treated with or without 1.5 μM antisense morpholino oligomer for 14 h, and then treated with various concentrations of IPA in the presence (●) or absence (■) of 0.3 μM of the antisense oligomer for 4 days. (A) Growth inhibition. (B) NBT reduction. (E and F) Morphologic changes. Data in Panels E and F represent cells treated with 25 μg/ml IPA for 4 days. The effects of antisense oligomer on S100P (C) and CD11b (D) mRNA expression are shown. Cells were treated with 0.5 μM antisense phosphorothioate oligonucleotides for S100P for 12 h, and then cultured with or without 25 μg/ml IPA for 1 (C) and 3 (D) days. (D) Cells treated without (●) or with 0.5 μM the antisense oligomer (■) or missense oligomer (▲). Values are means±S.D. of three separate experiments.

al. [19]. The present results about genes up-regulated by ATRA are compatible with previous reports [19,20]. Although CN-A greatly up-regulated S100P mRNA, the general gene profile was similar to that with ATRA rather than that with IPA. While both CN-A and VD3 induce cells to differentiate into monocytes/macrophages, CN-A up-regulated the expression of many genes whereas VD3 up-regulated a few genes. The two genes that were the most up-regulated by VD3 were arachidonate 5-lipoxygenase and prostaglandin-endoperoxide synthase 1, which suggests that the up-regulation of these genes is important in VD3-induced differentiation. However, these genes were not significantly up-regulated in cells treated with IPA. These results indicate that the gene profile of cells treated with IPA was completely different from those of cells treated with other compounds, suggesting a different mode of action. In comparing the up-regulated gene profiles, there were few down-regulated genes and the gene profiles were similar, suggesting that the down-regulation of gene expression is less important during differentiation induced by these compounds. Transcription factor Dp-2 was constantly down-regulated by all the inducers we tested. Dp-2 is a heterodimeric partner of E2F transcription factor that regulates the expression of genes at the G_1/S boundary [21]. The down-regulation may contribute the growth arrest associated with differentiation in the cells.

One of the least-studied members of the S100 family is S100P, which was initially purified from the placenta [8,9]. The sequential expression of different S100 proteins has been observed in esophageal epithelial cells during their differentiation [22]. S100P is transiently expressed during the early stage of differentiation, suggesting that it plays a role in normal development. There is also considerable evidence that S100P plays a role in cancer [23–26]. S100P stimulated NIH3T3 cell proliferation, and this effect correlated with an increase in the activation of MAPKs [27]. S100P-stimulated MAPK activation was profound and prolonged, unlike the effects of many growth factors, which produce a rapid and transient increase in MAPK activity. Several inducers of differentiation have been shown to cause MAPK activation in a similar prolonged manner. Among the inducers, IPA was the most potent activator of MAPK in

HL-60 cells. This activation is necessary for IPA-induced differentiation (Fig. 6). S100P expression has been observed in various solid tumor cells including breast, colon, and pancreatic cancers [23–25]. Furthermore, S100P expression has been correlated with decreased survival in patients with lung cancer [26]. However, normal peripheral neutrophils strongly express S100P mRNA, whereas little, if any, is expressed by leukemia cells from acute myeloid leukemia (AML) patients [28]. A recent report revealed that a gene-expression profile based on 133 genes predicted the clinical outcome across a cytogenetic risk group in AML. Among these 133 genes, the S100P gene was in the group that predicted a good prognosis [29]. Furthermore, a study on the expression profiles of neutrophil- and macrophage-specific genes indicated that S100P is a neutrophil-specific gene [30]. Partial blocking of S100P expression almost completely suppressed the morphologic differentiation of HL-60 cells treated with IPA (Fig. 7). These results suggest that S100P plays an important role in granulocytic differentiation. Despite this evidence that S100P may play an important role in granulocytic differentiation, its effects on cell function are unknown.

The up-regulation of S100P mRNA in HL-60 cells is immediately induced by IPA, CN-A and MJ. However, none of the other inducers we tested effectively induced S100P mRNA expression within 24 h. Interestingly, these three inducers are known as plant growth regulators [3,18]. An inverse correlation was found between DNA methylation and the expression level of mRNA for S100P in a large panel of pancreatic cancer cell lines [31]. Treatment with a combination of 5-aza-2′-deoxycytidine and tricostatin A resulted in the synergistic induction of S100P mRNA in a pancreatic cancer cell line where the gene was methylated [31]. These drugs are known to enhance the differentiation of myeloid leukemia cells [3,32–34]. IPA might alter the transcriptional activity of S100P promoter by its demethylation. Further research will be needed to better understand the importance of S100P in granulocytic differentiation.

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